Regulation of Human NK Cells by Cytokines and by Exposure to Bacterial Toxins Valinomycin and Cereulide

Auli Paananen

Department of Applied Chemistry and Microbiology
Division of Microbiology
and
Department of Pathology
Haartman Institute
University of Helsinki
Finland

Academic Dissertation in Microbiology

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in Auditorium 2 at the Viikki Info-center, Viikinkaari 11, on June 4th, at 12 o'clock noon.

Helsinki 2004

Supervisors:

Professor Dr. Mirja Salkinoja-Salonen
Department of Applied Chemistry and Microbiology
Division of Microbiology
University of Helsinki
Finland

Doc. Tuomo Timonen
Department of Pathology
Haartman Institute
University of Helsinki
Finland

Reviewers:

Professor Olli Silvennoinen
Institute of Medical Technology
University of Tampere
Finland

Professor Mikael Skurnik
Department of Bacteriology and Immunology
Haartman Institute
University of Helsinki
Finland

Opponents:

Docent Ove Erikson
Department of Biochemistry
Institute of Biomedicine
University of Helsinki
Finland

Docent Mieke Uyttendaele, Ph.D.
Food Microbiology and Applied Biological Services
University of Ghent
Belgium

ISSN 1239-9469
ISBN 952-10-1073-8
ISBN (e-thesis) 952-10-1074-6

Front cover: SEM picture of valinomycin treated NK cell

Cosmoprint OY, Helsinki, Finland

To my family

Contents:

List of original papers

I. Matikainen S., A. Paananen, M. Miettinen, M. Kurimoto, T. Timonen, I. Julkunen, T. Sareneva. 2001. IFN-α and IL-18 Synergistically Enhance IFN-γ Production in Human NK Cells: Differential Regulation of Stat4 Activation and IFN-γ Gene Expression by IFN-α and IL-12. Eur J Immunol 31:2236-2245

II. Paananen A., Mikkola R., Sareneva T., Matikainen S., Andersson M., Julkunen I., M.S. Salkinoja-Salonen, Timonen T. 2000. Inhibition of Human NK Cell Function by Valinomycin, a Toxin from Streptomyces griseus in Indoor Air. Infect Immun 68:165-169

III. Paananen A., R. Mikkola, T. Sareneva, S. Matikainen, M. Hess, M. Andersson, I. Julkunen, M.S. Salkinoja-Salonen, T. Timonen. 2002. Inhibition of Human Natural Killer Cell Activity by Cereulide, an Emetic Toxin from *Bacillus cereus*. Clin Exp Immunol 129:420–428

IV. Paananen A., K. Järvinen, E. Hölttä, T. Sareneva, M.S. Salkinoja-Salonen, T. Timonen. 2003. Valinomycin Induces Caspase Dependent and Independent Apoptosis of Human NK Cells. Submitted

The author's contribution

I. Auli Paananen was responsible for the experimental work on NK cells and wrote the paper together with the other authors.

II. Auli Paananen wrote the paper and is the corresponding author. She also planned and carried out the experimental work. Raimo Mikkola and Maria Andersson carried out the bacterial culturing and purification of the toxins. Ilkka Julkunen, Timo Sareneva and Sampsa Matikainen were the interleukin experts and carried out some of the ELISA assays.

III. Auli Paananen wrote the paper and is the corresponding author. She also planned and carried out the experimental work. Raimo Mikkola and Maria Andersson carried out the bacterial culturing and purification of the cereulide. Raimo Mikkola carried out the mass analysis of cereulide. Timo Sareneva and Sampsa Matikainen were the interleukin experts along with Ilkka Julkunen. Michael Hess prepared the TEM pictures.

IV. Auli Paananen wrote the paper and is the corresponding author. She also planned and carried out the experimental work. Kristiina Järvinen and Erkki Hölttä carried out the caspase-3 measurements. Timo Sareneva was the interleukin expert.

Abbreviations

AIF	apoptosis-inducing factor
Apaf-1	apoptosis-activating factor 1
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
CAD	caspase-activated DNAse
DDT	di(para-chloro-phenyl)-trichloroethane
DMSO	dimethylsulfoxide
endo-G	endonuclease G
ESI-MS	electrospray ionization-mass spectrometry
ESI-MS/MS	electrospray ionization-tandem mass spectrometry
FADD	Fas-associated death domain protein
FasL	Fas-ligand
GM-CSF	granulocyte macrophage colony-stimulating factor
HLA	human leukocyte antigen
ICAD	inhibitor of caspase-activated DNAse
IL	interleukin
IFN-γ	interferon gamma
JC-1	5,5', 6,6'-tetrachloro-1,1', 3,3' tetraethylbenzimidazolylcarbocyanine iodide
K_{ow}	the octanol-water partition coefficient
KIR	killer cell immunoglobulin like receptor
LOD	limit of detection
NK cell	natural killer cell
MHC	major histocompatibility complex
$\Delta\psi_m$	mitochondrial transmembrane potential
PBL	peripheral blood lymphocytes
PCB	polychlorinated biphenyls
S.E.	standard error
SEM	scanning electron microscope
Stat	a signal transducer and an activator of transcription
subG1	subgenomic DNA peak
TNF	tumor necrosis factor
TEM	transmission electron microscope
Th1	type 1 T helper cell response
Th2	type 2 T helper cell response
Z-DEVD-FMK	a specific caspase-3 inhibitor
Z-VAD-FMK	general caspase inhibitor

Abstract

Valinomycin and cereulide are toxic cyclic ionophoric peptides produced by bacteria found in food and/or the environment. Valinomycin is produced by several species of *Streptomyces*, whereas cereulide is produced mainly by *Bacillus cereus*.

Valinomycin and cereulide act as carriers of potassium ions through biological membranes. They are highly lipophilic and will therefore passively penetrate into tissues and cells, causing a health hazard. These toxins are stable and no method is known to destroy them in food or the environment.

Cereulide is produced by certain strains of *B. cereus* frequently present in food, for example in rice and beans. Valinomycin is structurally related to cereulide but is produced by *Streptomyces griseus*, commonly isolated from moisture-troubled buildings. Occupants of these houses suffer from an excess of trivial infections.

This study deals with the effects of cereulide and valinomycin on one part of the human immune system, natural killer cells (NK cells), known to be involved in defence against microbial infections and malignant disease.

NK cells represent 5-20% of peripheral blood lymphocytes and are capable of killing abnormal cells without prior activation. Activated NK cells also secrete cytokines, such as interferon gamma (IFN-γ), to regulate other arms of the immune system.

Valinomycin and cereulide were toxic to normal human NK cells, as measured by the reduction of the NK activity, increased apoptosis, and diminished cytokine production. The toxic effect was seen with very low, physiologically relevant concentrations of bacterial toxins. To our knowledge, this is the first time that such toxin effects were shown with normal human white blood cells.

Exposure to the toxins changed the morphology of normal human NK cells and a cultured NK cell line. Vacuole formation was clearly observed with light microscopy and in transmission electron microscopy, and mitochondrial swelling was seen after cereulide or valinomycin treatment. In addition, the surface projections normally occurring on human NK cells became shorter and thicker, as shown by scanning electron microscopy.

IFN-α, one of the most abundant cytokines released by virus-infected cells, in combination with IL-18 induced stimulation of IFN-γ production of NK cells. Additionally IL-12, -15, and -18 synergistically induced IFN-γ production, and protected NK cells from the toxic damage of valinomycin or cereulide. Interestingly, T cells and monocytes were not as sensitive as NK cells to these toxins.

The present results demonstrated for the first time that resting NK cells express high caspase-3 activity. Valinomycin treatment did not change this activity. Valinomycin induced two pathways of apoptosis in the NK cells; one of which was the caspase-3-dependent 'classical' pathway of apoptosis, and the other was the caspase-3-independent 'non-classical' pathway.

1. Introduction

This thesis describes interactions of NK cells with their environment and with two toxins, valinomycin and cereulide. Valinomycin and cereulide are lipophilic cyclic peptides and ionophores, produced by bacteria. These ionophorical toxins may cause imbalance in cellular potassium equilibrium. Humans may become exposed to valinomycin and cereulide via everyday food or via indoor air, especially in buildings with dampness damage. People living or working in such buildings are reported to continuously suffer from respiratory infections [1, 2]. The introduction describes the three main elements of this study, the two toxins and human NK cells. It also introduces the physical and physiological environments where the immune defence cells operate and interact with intercellular messengers and toxic bioactive substances.

1.1 Potassium equilibrium in cells

Potassium ions are essential to living cells. Their transport is part of cellular signalling, creates electrical potentials in the cells, and also contributes to the cellular osmotic balance. Potassium ions are positively charged, and are transported through biological membranes by active transport systems like ATP sensitive Na^+-K^+ pumps or voltage gated channels, to maintain the cellular potassium equilibrium. Inside normal animal cells the K^+ concentration is 140 mmol/l, approximately 30 times higher than outside (4 mmol/l) [3].

1.1.1 Na^+-K^+ pumps

ATP dependent Na^+-K^+ pumps are present in the plasma membranes of all animall cells. These membrane-spanning proteins work as ATPases and antiporters, transporting three Na^+ ions out and two K^+ ions in to cells against their electrochemical gradients with the expense of ATP being hydrolyzed to ADP. These pumps use up to 70% of cellular ATP. The other important task of these pumps is to maintain the osmotic balance and stabilize the cell volume [3, 4]. (Figure 1)

1.1.2 Potassium channels

Ion channels allow ion leakage downstream of the ionic gradients through the membrane by special pores. These channels are ion-selective; i.e. they select for K^+ over Na^+ based on the specific ion dimension or on the hydrated ion size (Figure 2).

Ions can pass through these channels at a rate 10^5 higher when that conducted by transporters. High voltage stimuli open the gates of the potassium channels. After cessation of the stimuli the channels are inactivated and closed. This type of channel is typical for excitable cells such as nerve cells. There are also Ca^{2+}-activated K^+ channels in nerve cells, opened by elevated Ca^{2+} concentrations on the cytoplasmic side of the nerve cell membrane (Ca^{2+} can be elevated inside nerve cells because of voltage-gated Ca^{2+} channels). [4]

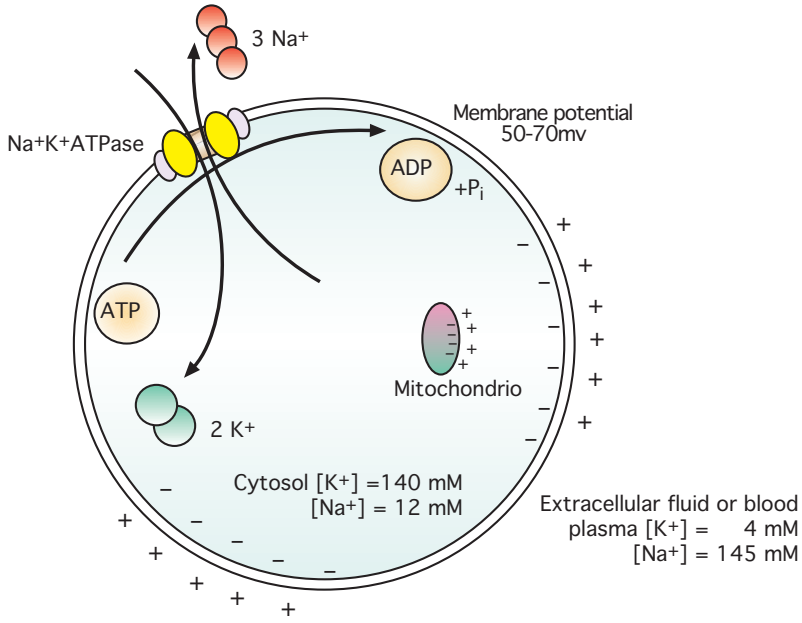


Figure 1. Na^+-K^+ ATPase and Na^+-K^+ equilibrium in an animal cell. The pump maintains the intracellular concentrations of potassium and sodium ions, and generates the transmembrane electrical potential. Modified from Nelson et al. [3]

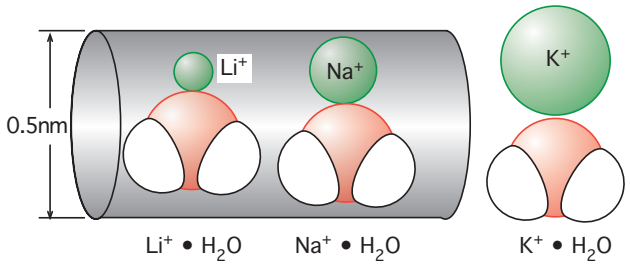


Figure 2. Sizes of hydrated cations, modified from Stryer [5].

1.2 K^+ ionophoric bacterial toxins

Bacteria can produce toxins to help them survive in certain environments. Bacterially produced ionophores can disrupt the human cellular Na^+-K^+ equilibrium by transporting K^+ ions downstream of their electrical or concentration gradients, which are maintained by membrane sodium-potassium pumps [4].

Several different taxa of microorganisms are known to produce K^+ ionophores (Table 1) [6, 7]. These are known to function as carriers or as channel formers [6]. Nonactin, nigericin, valinomycin, cereulide, and polyether ionophores are ion carriers whereas alamethicin and gramidicin form special ion transporting channels in the biological membranes [6, 8, 9] (Table 1).

Table 1. Various K^+ ionophores produced by microorganisms

Compound	Transported ions	Transport type	Producers and structure	Ref.
Valinomycin	K^+, Rb^+	Mobile carrier	*Streptomyces griseus, S. tsusimaensis, S. fulvissimus,* peptide	b
Nonactin	NH_4^+, K^+	Mobile carrier	*Streptomyces* sp.	c
Cereulide	K^+	Mobile carrier	*Bacillus cereus*, peptide	b
Nigericin	K^+,H^+	Mobile carrier	*S. hygroscopicus*, polyether	a
Salinomycin	K^+	Mobile carrier	*S. albus*, polyether	a, b
Lasalocid	K^+, Ca^{2+}	Mobile carrier	*S. lasaliensis*, polyether	a
Enniatin	NH_4^+, K^+	Mobile carrier	*Fusarium* sp., peptide	a, b
Narasin	K^+	Mobile carrier	*S. aureofacians*	a, b
Gramidicin	H^+, Na^+, K^+	Channel former	*Brevibacillus brevis*	b, c
Alamethicin	K^+, Rb^+	Channel former	*Trichoderma viride*	

a [6], b [7], c [9]

Some antimicrobial drugs act as ionophores. Examples of such antimicrobials, in everyday use in Europe and in the USA as additives in the animal feed industry, are the potassium ionophores narasin, salinomycin, and lasalocid. These three ionophores are used as coccidiostats, i.e. as prophylaxis towards certain unwanted gastrointestinal parasites (coccidiosis, caused by *Eimeria* sp). Salinomycin is also a growth promoter [8]. In Finland these three antimicrobials are used for poultry, but in some countries they can also be used for dairy and beef cattle and pigs [10-12].

In many EU countries, animal products (milk, meat, and eggs) are tested for antibiotic residuals. Amounts below the action levels (set by food safety authorities) are frequently reported [10, 13] (Table 2). Addition of lasalocid to poultry feed in Finland was interrupted in the spring of 2003 after two lasalocid positive eggs were found [13]. When narasin and salinomycin were tested for inhibition of boar sperm, as a test to

detect mitochondriotoxins, sperm motility inhibition was observed exposure concentration of 50 ng/ml. Cereulide, valinomycin, and lasalocid were the most toxic to sperm, inhibiting the sperm motility with exposure concentrations of 0.5-2.5 ng/ml (unpublished results of Hoornstra) [7].

Table 2. Potassium ionophoric growth promoters and coccidiostats used in the livestock industry

Ionophore	Countries	Animals	LOD (limit of detection)	Action level	Positive findings	Ref.
Lacalocid/ lasalocid	EU nations	Poultry, eggs	25 µg/ kg/ Finland/ EU	Positive detection	2 egg samples / Finland 2002	a, b, c
	USA	Cattle			Also in Austria, United Kingdom, Belgium, and Germany	
Salinomycin	EU countries USA	Poultry, ducks, eggs, pigs	0,4-24,8 µg/ kg/ Denmark 10 µg/ kg/ Finland/ EU	25 µg/ kg/ Denmark positive detection/ EU	Denmark	a, b, c, d
Narasin	EU countries USA	Poultry, ducks, eggs	10 µg/ kg/ Finland/ EU	positive detection	Sweden	a, b, c

a [13], b [10], c [12], d [8]

Two ionophoric bacterial toxins, valinomycin emitted by strains of *Streptomyces griseus* into indoor air, and cereulide produced by strains of *Bacillus cereus,* were investigated in the present study. Both toxins are highly effective and mobile carrier ionophores selective for K^+ over Na^+, at a ratio of 1000:1 [9] [14]. Both toxins are uniporters, carrying only one ion at a time across membranes [9].

1.2.1 Valinomycin produced by *Streptomyces* species

Valinomycin belongs to the peptide antibiotics, synthetisized by a multienzyme complex in different species of *Streptomyces* such as *S. levoris, S. tsusimaensis, S. lividans, S. fulvissimus, S. griseus,* and *S. coelicolor* [7, 15]. Valinomycin has been known for a long time. It is a cyclic ionophoric dodecadepsipeptide, transporting potassium ions selectively through mitochondrial membranes [9, 16] (Figure 3). This selectivity for potassium ions is due to the exact fit of the cavity inside the cyclic shape of the valinomycin molecule and the ability of different amino- and 2-hydroxyacids to hold K^+ there [17]. Cereulide is chemically closely related to valinomycin but differs in biological activity; it causes emetic illness in humans, while valinomycin does not [18].

Valinomycin is stable. Its biological activity is not destroyed by cooking, heating or treating by extreme pH. It is also very lipophilic (log K_{ow} = 5,99), which enables passive penetration through biological membranes, thus increasing toxicity [1, 14]. This penetration causes K^+ ions to flow downstream the electrical potential, from cytoplasm to mitochondria, and depolarization of mitochondria and hyperpolarization of the plasma membrane [7].

Valinomycin has been shown to induce apoptosis in numerous human and murine cell lines, such as BAF3 and P-815, at relatively high exposure concentrations (µg/ml). Evidence shows how valinomycin dissipates the mitochondrial electric transmembrane potential and induces collapse of the mitochondrial outer membrane, which then initiates apoptosis [19, 20]. Inai et al. [21] revealed that valinomycin collapsed the mitochondrial electric transmembrane potential of rat ascites hepatoma cells (AH-130) and activated caspase-3 like proteases, initiating apoptosis at exposure concentrations as low as 100 ng/ml. Other investigators also reported apoptosis in rat cells and different cell lines of human origin, but induction of apoptosis in normal cells by valinomycin has not been described before.

1.2.2 Cereulide produced by *Bacillus cereus*

Cereulide is a recently found peptide toxin, structurally related to valinomycin [24, 25] (Figure 4). It transports potassium ions and is highly lipophilic (log K_{ow} = 7,46) [1, 14].

Bacillus cereus is a frequent contaminant of everyday food, especially of starchy foods like rice but also dairy products, eggs, vegetables, and cereal products [26, 27]. Cereulide producing strains of *B. cereus* are reported in rice and pasta foods, but only seldom in dairy products [28, 29]. There are cases in which skin contact with *B. cereus* contaminated foodstuff resulted in emetic disease. Direct penetration of the toxin through the skin was suggested [30].

B. cereus, like other *Bacillus* species, forms heat resistant spores which are difficult to destroy. It is mesophilic and some strains are psychrotolerant, growing between +4 and +7°C. *B. cereus* strains can produce numerous different toxins, for example hemolysins, enterotoxins, and phospholipases, of which cereulide is one of the

most harmful [28]. The reason is the same as with valinomycin, it is highly lipophilic, chemically stable, and impossible to inactivate [1]. Only specific classes of *B. cereus* strains can produce cereulide. The diarreal enterotoxin producer strains do not produce cereulide [26].

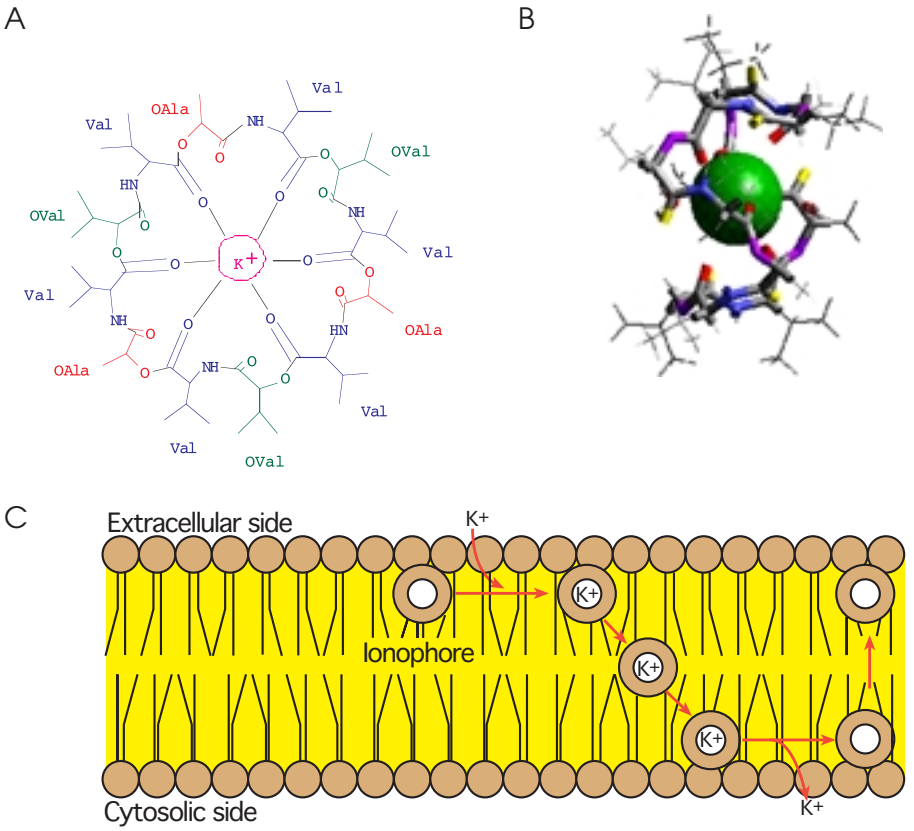


Figure 3. A. The structure of valinomycin molecule associated with K^+ ion. Modified from Ovchinnikov [22]. The hydrophilic groups in the cavity of valinomycin molecules hold K^+ ions during transportation [9]; B. Model of valinomycin molecule with the K^+ ion [23]; C. Model of K^+ ion transportation across a biological membrane by valinomycin. Modified from Devlin [9].

The production of cereulide was reported to be increased when food was stored at a temperature of over 30°C. At 35°C *B. cereus* strain NC7401 produced over 2000 ng/g cereulide in boiled rice within 24 hours [31]. Finlay et al. [32] investigated skim milk and concluded that the toxicity of *B. cereus* F4810/72 extracts to Hep2 cells was highest at temperatures from 12 to 15°C. It was recently shown that

cereulide concentrations may reach 300 to 600 ng/g in bakery products within 4 days of storage at 21 to 23°C. The normal shelf life of bakery products ranges from 4 to 75 days, depending on the product. Cool storage (≤ 8°C) was reported to prevent cereulide production. Bakery products containing rice or rice starch accumulated as much as 5.5 µg/g of cereulide when a cereulide producing *B. cereus* strain was present. White bread stored under similar conditions contained only 0.006 µg/g of cereulide [33].

Food poisoning by *B. cereus* may even be fatal, and causes emetic illness all over the world. Because *B. cereus* poisonings are not reportable diseases, the published values of *B. cereus* related cases, 1 to 47% of all food poisonings, are inaccurate, underestimations or overestimations, and even include those caused by viruses [27, 34].

Cereulide causes nausea and vomiting in primates and in the shrew *Suncus murinus* [25, 35, 36]. Yokoyama et al. [37] showed that rodents were insensitive to orally given cereulide, but the intraperitoneally injected synthetic cereulide caused the same kind of morphological changes as in human. Pathological changes were observed in the mice livers 2 to 3 days after cereulide treatment, but in the survivors changes disappeared after 4 weeks as measured by serum hepatic enzyme levels and histopathological analyses.

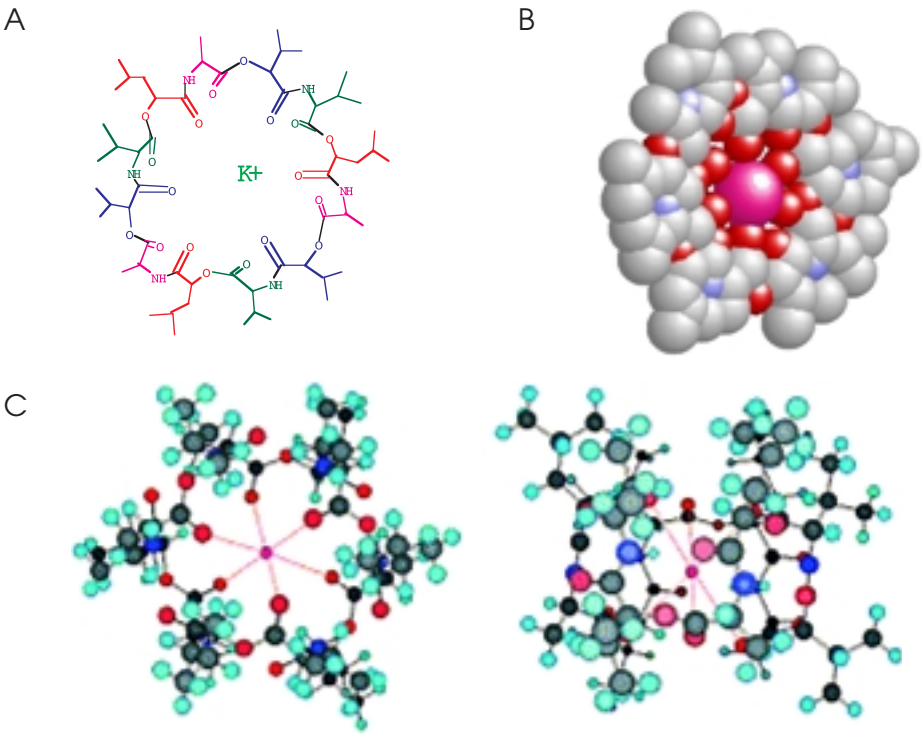


Figure 4. Structures of cereulide molecules interacting with K^+ ions. A , B [38] and C [18]

Harmful effects of cereulide are clearly visible at the cellular level. Cereulide causes mitochondrial swelling in HEp-2 cells, and hence is termed vacuolation factor [39]. Similar to valinomycin, the K^+ ionophoric property of cereulide causes the mitochondria to swell. This mitochondrial swelling may result from inflow of K^+ ions from the cytoplasm along the electrical gradient towards the negatively charged mitochondrial interior [14, 24]. This mechanism is supported by the observation that the cytoplasm of sperm cell became hyperpolarized in response to cereulide exposure — reflecting the loss of positively charged K^+ from the cytoplasm [7].

1.3 NK cells

NK cells form the third major subpopulation of lymphocytes, comprising 5 to 20% of peripheral blood lymphocytes. Upon activation NK cells migrate into tissues. For example, during inflammatory conditions they are found in lung and liver parenchyma [40]. NK cells originate from a common precursor with T cells, but do not require thymic influence for maturation. Most NK cells express a $CD3^-CD16^+CD56^+$ phenotype, but are heterogeneous, and at least 48 different subtypes have been described [41].

Morphologically, NK cells are medium to large sized granular lymphocytes with a high cytoplasm to nucleus ratio like activated T lymphocytes [42]. The nucleus is often kidney shaped. The cytoplasm of NK cells contains a relatively abundant number of mitochondria compared to, for example, T cells [40].

1.3.1. Receptors

NK cells express many different activating and inhibitory receptors, which maintain the delicate balance of positive and negative signals to the cytolytic machinery (Figure 5). The ligands for these receptors are classical and nonclassical MHC I molecules. The killer cell immunoglobulin like receptor (KIR) family recognizes and binds the classical MHC I molecules. Some of these receptors mediate inhibition of NK cell cytotoxicity, while the functions and ligands of other receptors are still unknown [43]. The nonclassical MHC I molecule HLA-E is recognised by the lectin-like CD94/NKG2 receptor family. Both receptor families contain both inhibitory and stimulatory forms.

An individual NK cell can simultaneously express several activating and inhibitory receptors. Most NK cell receptors are encoded by the NK-gene complex and leukocyte-receptor complex [44]. Other surface molecules, such as CD11a/CD18, 2B4, CD2, and CD69, may induce or modulate NK cell functions, too [44]. (Figure 5).

1.3.2. Functions

NK cells have many important functions. They kill abnormal cells without prior activation, including tumor cells, virus- and other pathogen-infected cells. They recognise these target cells through their surface receptors (Figure 5B). The lytic machinery of NK cells operates mainly through a pore-forming protein, perforin, and the proteolytic granzymes A and B [40, 42].

One NK cell is able to kill more than one target cell, although this ability is limited. After a certain number of killing cycles, NK cell apoptosis is induced through the killer receptors [45].

Another main function of NK cells is the protection against viral infections. As NK cell deficient patients usually suffer from severe herpes virus infections, it has been suggested that NK cells are especially effective against this group of viruses [46].

Furthermore, NK cells produce cytokines such as interferon gamma (IFN-γ) (Figure 6), tumor necrosis factor-α (TNF-α), lymphotoxin, IL-3, -5, -8, -10, -13, and granulocyte macrophage colony-stimulating factor (GM-CSF), after being stimulated by various cytokines [46]. NK cells produce these cytokines to regulate other cells in the immune system and also for autocrine regulation. For example, IL-2 and IL-15 induce proliferation of NK cells, and IL-12 and IFN-γ stimulate NK cell cytokine production [47].

1.4 Cytokines and interferons in the NK cell system

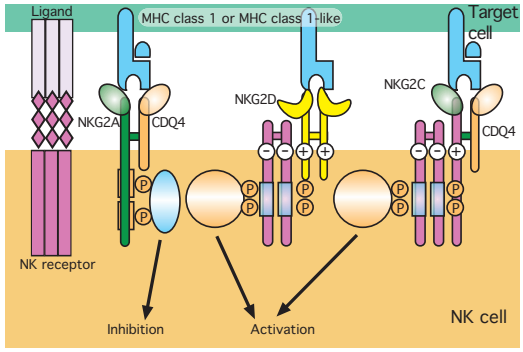
Cytokines are part of the soluble mediators in the regulation of immune defence. The cytokines regulate differentiation, proliferation and also cytokine production of various cell types. Cytokines are small, non-structural, intercellular regulatory proteins. Interleukins and interferons are central components of the cytokine system. At least 29 interleukins and four interferons are currently known, and the number of interleukins increases continuously [48-52].

The pattern of interferon and cytokine production by NK cells facilitates the Th1 response (CD 4^+ T helper cell response), which means that NK cells produce Th1-type cytokines, like IFN-γ after stimulation by IL-12 and IL-15. At same time they do not produce Th2 (type 2 T helper cell) response cytokines, such as IL-4. For the Th2 response, NK cells need a priming IL-4 stimulus. Th2 response is known to be associated with allergy. The early Th1 response begins with phosphorylation of a signal transducer and an activator of transcription 4 (Stat 4), whereas the Th2 response begins with Stat 6 phosphorylation [53, 54].

1.4.1 IFNs and GM-CSF on NK cells

The IFN-γ stimulates immune cells to mediate immune defence, especially Th1 response, against intracellular infections like those by viruses. Therefore, impairment of IFN-γ production often leads to decreased resistance to viral infection [42].

A



B

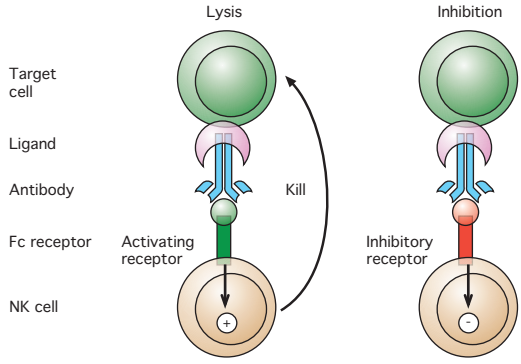


Figure 5. A. Inhibitory and stimulatory receptors of NK cells. B. Mechanisms of inhibition and activation of NK cell. Modified from Yokoyama et al.[44].

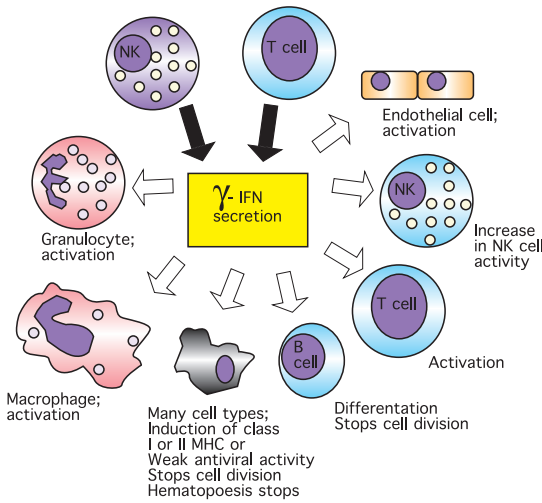


Figure 6. IFN-γ production by NK and T cells. IFN-γ is a regulator of many different cell types, including NK and T cells. Modified from Hunt [60].

After human cells are infected by viruses they produce type I interferons (IFN-α/β), which enhance NK cell killing ability towards virus-infected cells [55]. IFN-α also activates human Th1-type immune responses through Stat4, which leads to IFN-γ gene expression in T cells [56]. After viral infection, macrophages produce IFN-α/β and IL-18, which together can enhance IFN-γ gene expression in T cells [57].

GM-CSF is another cytokine produced by NK cells after stimulation [58]. GM-CSF directs granulocyte and macrophage colonies towards maturation. Monocytes and fibroblasts also produce GM-CSF [59].

1.4.2 IL-12

IL-12 is member of a small heterodimeric cytokine family with two other recently discovered interleukins -23 and -27 [49]. IL-12 is produced by phagocytic cells and dendritic cells after microbial infection. Many cell types express IL-12 receptors, but the receptors are most abundant on NK cells and on activated T cells. IL-12 induces proliferation, cytotoxicity, and IFN-γ production [61, 62]. IL-12 works synergistically with IL-18 in the induction of NK and T cell proliferation [63, 64].

Table 3. NK cells and selected cytokines

Cytokines affecting NK cells		The resulting effect of NK cell functions
Effector cell	Produced cytokine	
T cells	IL-2[a]	Stimulates proliferation and differentation
monocytes/ macrophages	IL-12[b]	Stimulates Th1 differentation, inhibits Th2 differentation
monocyte/macrophage, dendritic cells human bone marrow stromal cells	IL-15[c,a]	Stimulates NK cell proliferation and differentation
macrophage dendritic cells	IL-18[d]	Stimulates maturation, cytokine production, and cytotoxicity
T helper cells	IL-21[e]	Enhances cytolytic activity
NK and T cells	IFN-γ[f]	Facilitates Th1 response
NK cells	GM-CSF[g]	Stimulates granulocyte and macrophage colonies growth and differentiation

a [48], b [62], c [65], d [50], e [66], f [42], g [59]

1.4.3 IL-15 and IL-2

The most important function of IL-15 is to initiate the differentiation of NK cells and T cells from progenitor cells in the bone marrow [65]. IL-15 may be the critical interleukin for the in vivo survival of the NK cells [67, 68]. IL-15 shares a part of its heterotrimeric receptor with IL-2. IL-2 is homologous with IL-15, with which it shares many functions. The difference is that IL-15 more broadly enhances NK cell functions than does IL-2, and IL-15 has a wider tissue distribution [65]. IL-15 is produced by many tissues and cells, such as monocytes, macrophages, dendritic cells, and bone marrow stromal cells [65].

1.4.4 IL-18

IL-18 is another important cytokine regulating NK cells. IL-18 is produced, for example, by macrophages, dendritic cells, and keratinocytes. In addition to NK cells, many other cells also express IL-18 receptors. The main function of IL-18 is to stimulate response against microbial infection. IL-18 activates and facilitates many functions of NK cells, such as maturation, cytotoxicity, and cytokine — especially IFN-γ — production [50, 63]. IL-18 is often a co-stimulatory cytokine along with IL-15 or IL-12. Synergy of these cytokines increases the rate of IFN-γ production and proliferation of NK cells [63, 64].

1.4.5 IL-21

IL-21 is a recently discovered cytokine produced by activated $CD4^+$ T cells [51]. T, B, and NK cells express IL-21 receptors. This interleukin may elicit inhibitory or stimulatory effects on NK cells. It can stimulate cytolytic activity alone or synergistically with IL-15, and expand the NK cell pool of the bone marrow or of peripheral blood. High doses of IL-21 inhibit IL-2 induced NK cell proliferation whereas low doses may stimulate it [66]. According to Strengell et al. [69] IL-21 acts synergistically with IL-15 and IL-18 in stimulating NK and T cell IFN-γ production.

1.5 Caspase-3

Caspase-3 is a cysteine aspartate protease. Caspase-3 has traditionally been regarded as a key regulator of apoptosis. Recently however, part of apoptosis has been noticed to be caspase-3-independent. The two apoptotic mechanisms, caspase-3-dependent and caspase-3-independent, may operate simultaneously [70]. Caspase-3 activity is also involved in the proliferation of T cells [71].

1.5.1 Caspase-3 dependent apoptosis

Cells can be destroyed by necrosis, or they can die through apoptosis i. e. programmed cell death. In apoptosis the cells themselves launch a cascade, which causes typical morphological apoptotic changes to cells, such as DNA degradation, cell shrinkage, and membrane blebbing [72]

The 'classical' caspase-dependent route includes two pathways, the extrinsic and intrinsic ones. The extrinsic pathway is triggered by death receptors such as Fas or TNF receptors. It is mediated by Fas-associated death domain protein (FADD), which cleaves initiator caspase-8 to its mature form. Caspase-8 cleaves procaspase-3 to produce mature caspase-3 [72, 73]. Caspase-3 cleaves the inhibitor of caspase-activated DNAse (ICAD) from the CAD:ICAD complex, allowing caspase-activated DNAse (CAD) to cleave chromosomal DNA [74].The intrinsic pathway of apoptosis cascade begins from the mitochondrial membrane disruption. This leads to the leakage of cytochrome c from the mitochondria and to the consequent clustering of apoptosis-activating factor 1 (Apaf-1) with caspase-9. This cluster is called an apoptosome. The apoptosome converts the precaspase-3 into its mature form [72, 73]. (Figure 7).

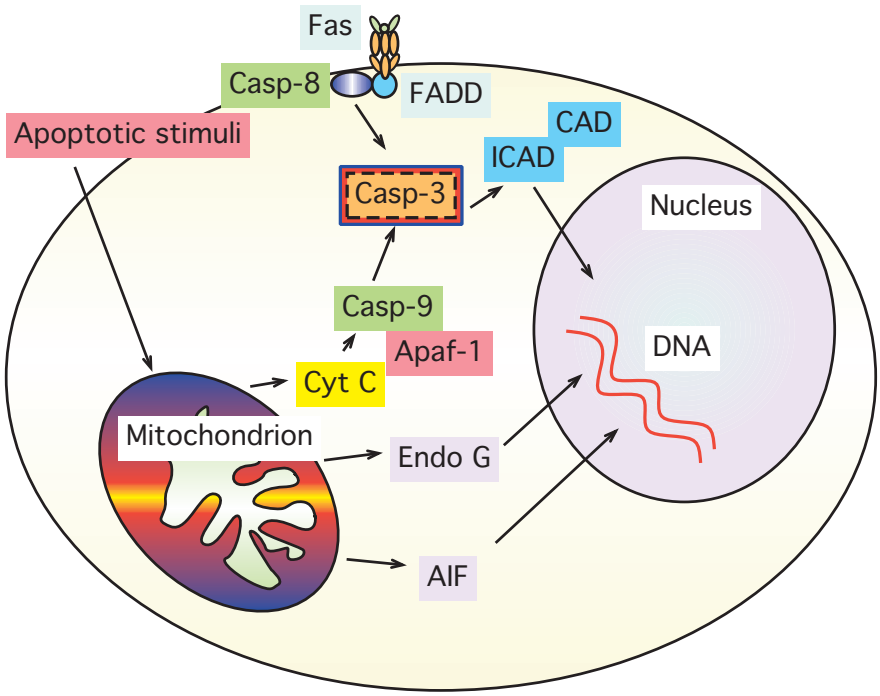


Figure 7. Apoptosis pathways. The two caspase-dependent pathways; the extrinsic pathway is triggered via Fas, and the intrinsic one is triggered by apoptotic stimuli. Apoptotic stimuli can also trigger caspase-independent apoptosis when AIF or endo G from damaged mitochondria initiate apoptosis.

1.5.2 Caspase-3 independent apoptosis

Parallel to caspase-3-dependent apoptosis, there exists another type of programmed cell death, which does not require caspases. This is generally called the 'non-classical' pathway. This type of apoptosis has been described to occur in many cell types, such as human neutrophils, T cells, COS, U937, Rat-1, and rat hepatoma 512tc cells, among others [70, 75-77]. In many cases apoptosis is initiated by apoptosis-inducing factor (AIF), which is released from a damaged mitochondrion. This initiator does not trigger the caspase cascade nor regulate it, but instead triggers caspase-independent apoptosis [76, 78]. Recently Li et al. [79] found a new apoptosis factor from mitochondria, which can induce caspase-independent apoptosis. This endonuclease G initiates DNA fragmentation independently from caspase-3.

2. Aims of this study

The specific goals were to:

2.1 Elucidate details of interleukin regulation of central NK cell functions.

2.2 Measure the effects of the potassium ionophoric peptide toxins, valinomycin and cereulide, on NK cell functions such as cytotoxicity, IFN-γ production, and apoptosis.

2.3 Investigate the apoptotic effect of these toxins on NK cells.

2.4 Discuss the consequences of human exposure to microbially produced potassium ionophores like cereulide, valinomycin, lasalocid, salinomycin and narasin.

3. Materials and methods

Materials and methods used in this study are listed in table 4.

Table 4. The methods used in this study

Assay	Described in papers	Reference
Purification of peripheral blood mononuclear cells (PBLs)	I, II, III, IV	
Cell cultures (NK-92, HeLa, HepG2, and K-562)	I, II, III, IV	[80]
Cytokine activation of NK and T cells	I, II, III, IV	
^{51}Cr-release cytotoxicity assay	I, II, IV	[81]
Electron microscopy (SEM and TEM)	I, II	[82]
NK cell exposure to bacterial toxins	I, II, IV	
Annexin-V-apoptosis assay	I, II, IV	[83]
Fluorescence JC-1 staining assay	II	[84]
IFN-γ ELISA assay	I, II, III, IV	
GM-CSF ELISA assay	I	
Purification of cereulide from *Bacillus cereus*	II	
Identification of cereulide by specific mass ions by ESI-MS and ESI-MS/ MS	II	
RNA isolation and analysis	III	
DNA isolation and analysis	III, IV	[83]
Caspase-3 assay	IV	

4. Results and discussion

4.1 The effects of different cytokines on NK cells

The effects of IFN-α, IL-12, IL-15 and IL-18, applied separately or in various combinations on NK cell IFN-γ and GM-CSF productions were studied.

It is already known that in virus infected T cells IFN-α and IL-18 synergistically enhance IFN-γ production [57]. Furthermore, Sareneva et al. 2000 [85] reported that IFN-α and IL-12 induce IL-18 receptor gene expression in T cells and in NK cells.

In the present study, NK, T, and NK-92 cells were first stimulated either with IFN-α plus IL-18 or with IL-12 plus IL-18. The effects were then monitored by measuring the amount of IFN-γ production (by ELISA-assay) and the kinetics of IFN-γ mRNA synthesis (by Northern blotting).

Next, the effect of IL-12, -15 and -18 in various combinations on cytokine production of NK cells was studied. It was also examined how the two environmental bacterial toxins, cereulide and valinomycin, affected the interferon stimulated IFN-γ and GM-CSF production by NK cells.

4.1.1 NK cell responses to cytokine stimulation

The results in Paper I show that IFN-α and IL-18 enhanced IFN-γ production of purified resting human NK cells in a synergistic manner. Stimulation of NK cells by IFN-α or by IL-18 separately elicited only a low IFN-γ production, <100 pg/ml. When NK cells were stimulated with a combination of IFN-α and IL-18, the IFN-γ production was much higher, >2000 pg/ml. The combination of IL-12 and IL-18 also induced high IFN-γ production, up to >40000 pg/ml, as compared to stimulation with a single interleukin, resulting in the production of <1000 ng/ml. These results are shown in details in Figure 1, Paper III.

The difference between the effects of IL-12 plus IL-18 and IFN-α plus IL-18 stimulations was reflected in the kinetics of the IFN-γ production by NK cells. IFN-γ production induced by IFN-α plus IL-18 was highest at the 6 h time point and remained elevated until 20 h, as compared with IL-12 plus IL-18 induced stimulation, which continued to increase for up to 20 h (Figure 2A, Paper I). This difference in the kinetics was also seen in the expression level of IFN-γ mRNA. IFN-α when applied alone, stimulated the IFN-γ gene expression up to its peak value in 2 to 3 h. The IFN-α-induced IFN-γ mRNA level decreased rapidly, whereas the IL-12-induced IFN-γ mRNA levels continued to increase during the 10 h experiment. (Figure 4, Paper III). All effects observed in the IFN-γ production by NK cells were also seen in T cells (Paper I).

The results described above indicate that immune defence involves more than one efficient pathway to produce needed IFN-γ to counteract infections by bacteria or by viruses. The production of IFN-γ by NK cells or T cells is lower in viral infections (shown by IFN-α plus IL-18) than in bacterial infections (shown by IL-12 plus IL-18). This may suggest that viruses are more susceptible than bacteria to IFN-γ-induced immune defence or maybe γ-IFN is needed for much lower concentrations to induce the necessary defence against viral infections.

The present results demonstrated the synergism of IL-12, -15 and -18 in IFN-γ and GM-CSF production by NK cells. IL-15 alone stimulated IFN-γ production up to 20 000 pg/ml, whereas IL-18 induced IFN-γ production was around 1000 pg/ml. The combination of these two interleukins, however, was synergistic, increasing the IFN-γ production up to 40 000 pg/ml. The influence was similar on GM-CSF: the synergy of IL-15 and IL-18 made NK cells to produce at least twice as much GM-CSF as compared with either of these interleukins alone. (Figure 5, Paper II).

IL-12 alone stimulated only a low level of IFN-γ production. However the simultaneous effect of the combinations of IL-12 with IL-15 or IL-12 with IL-18, stimulated γ-IFN production significantly ($p \leq 0.05$). Simultaneously adding all three interleukins resulted in IFN-γ production that was at least twice as high as that observed with the combination of two interleukins only, as shown in Figure 7, Paper III.

In vivo conditions are difficult to imitate *in vitro,* but the results do suggest that tuning of the immune system to its maximal capacity requires the combination of several cytokines. The optimal cytokine combination is probably dictated by the type of infection or noxious stimulation.

4.1.2 Effects of valinomycin and cereulide on cytokine stimulation

Various environmental exposures can harm immune functions. Here it was shown how two bacterial K^+ ionophoric toxins, valinomycin and cereulide, hampered IFN-γ and GM-CSF production by NK cells. NK cells were first exposed for 2 h to valinomycin or cereulide, simulating a situation where toxin-producing bacteria infect the host. The IFN-γ production by toxin-treated NK cells was strongly reduced when they were incubated with any of the interleukins alone; only modest IFN-γ production was seen. But interestingly, when the toxin-treated NK cells were incubated with a combination of interleukins, a substantial recovery of IFN-γ production was evident. The same result was seen also on GM-CSF production. (Figure 5, Paper II).

Cereulide was equally toxic as valinomycin to NK cells, as demonstrated by in the dramatic reduction of IFN-γ production by exposed cells. Recovery of IFN-γ production was efficient under influence of the combination of IL-15 and IL-18, and even more efficient when all three interleukins (IL-12, -15 and -18) was used to stimulate the NK cells after the toxin treatment (Paper III, figure 7).

NK cells were more sensitive to toxins than were T cells and monocytes. The toxic effect on T cells came later, after 72 h culturing, as compared to the 24 h when the NK cell had been exposed. Monocytes were not sensitive to valinomycin, or cereulide (Figure 7, Paper III).These results suggest that valinomycin and cereulide are toxic primarily to NK cells. The results also showed that cereulide was somewhat less toxic than valinomycin as measured by IFN-γ production by NK cells.

4.2 Toxin effects on the cytotoxic activity of NK cells

One main function of NK cells is the ability to destroy abnormal cells. The effect of valinomycin and cereulide on freshly isolated PBLs was investigated using the Cr-release assay with cultured K-562 as the target. This cancer cell line is especially sensitive to killing by natural killer cells. Valinomycin from two different origins were used. One valinomycin preparation was of commercial grade, purified from *S. tsusimaensis*, and the other was purified from a *S. griseus* strain isolated from a Finnish moisture damaged indoor environment. Considering the different species of producer bacteria, the two valinomycin preparations may have contained different impurities. Cereulide was purified from *Bacillus cereus* strains connected to food poisoning. The purity of PBLs was sufficient for the experiments because NK cells are the only cell type that kill target cells without prior activation.

4.2.1 Valinomycin effects

Valinomycin inhibited NK cell cytotoxicity efficiently as measured by the reduction in the percentage of cytotoxicity. The inhibition varied between 10 % and 60 %. The kinetics of this inhibition was fast, the maximal effect already seen at the 1 min time point. Spontaneous release (= in absence of NK cells) of chromium from valinomycin-treated K-562 target cells was similar to that from untreated K-562 target cells. The observed inhibition of NK cell killer activity depended on the dose of valinomycin. Small doses, 20 to 50 ng/ml, impaired killing by 10 to 30 %, and doses over 70 ng/ml, inhibited cytotoxicity at least 50 %. Interestingly, the inhibition always remained partial, even at high doses, 500 ng/ml (Figure 1, Paper II). The slight difference in the effects of the two valinomycin preparations may reflect the fact that they originated from different species and the impurities may have interfered with the biological effect of the toxins. The actual concentration of valinomycin may also have differed slightly between the two preparations, but only slightly as there was no reflection observed in the shapes of the dose response columns in Figure 1, Paper II.

The inhibition remained constant over the 4 h of testing, shown in Figure 8. The maximal inhibition was reached with an exposure concentration of 70 ng/ml of valinomycin. No further increase in toxicity was seen with higher concentrations (Figure 5 and Figure 1, Paper II).

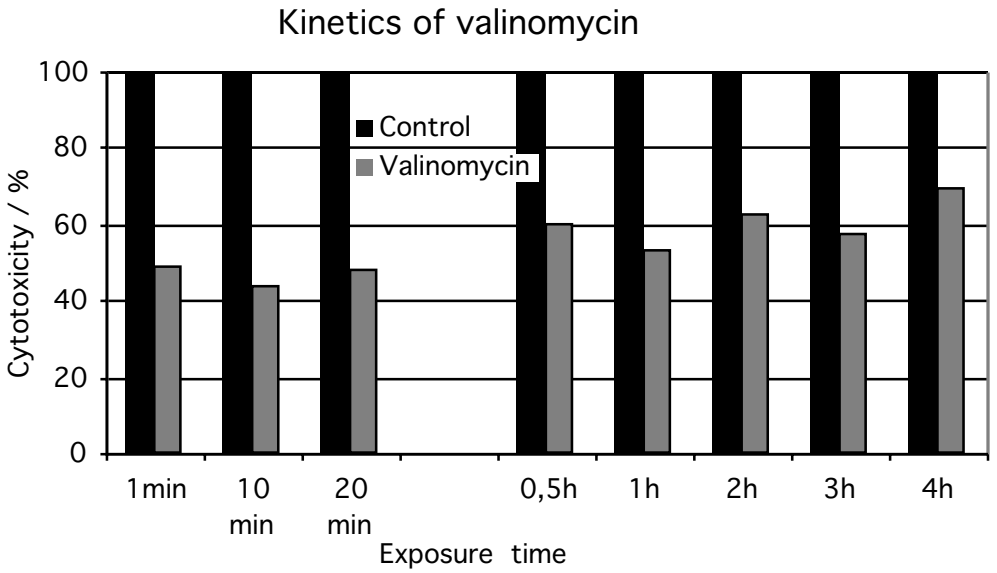


Figure 8. Kinetics of cytotoxicity inhibition of PBLs after exposure to 50 ng/ml valinomycin. Each value represents an average of those obtained from 6 individual donors. Group I (PBLs of 6 donors) was exposed for 1 to 20 min and group II (PBLs of 6 donors) for 0.5 to 3 hours.

Interleukin treatment before or after toxin exposure was observed to prevent the inhibition of NK cell cytotoxicity, as seen in Figure 9. According to JC-1 staining results (Figure 13), the mitochondrial membrane potential of the interleukin-stimulated, valinomycin-treated cells was affected as much as the mitochondrial membrane potential of the noninterleukin- stimulated, valinomycin-treated cells. This indicates that interleukins did not protect the NK cells against the loss of mitochondrial transmembrane potential, leading to the onset of apoptosis, by the toxins. The fact that the interleukins activated the cytotoxicity of the NK cells, even after the toxin exposure, means that the cytotoxic machinery of NK cells probably utilized the remaining ATP pool, which is present in the effector cells (Table 1, Paper IV). According to the results shown in table 1, paper IV the toxin exposure reduced the ATP content of NK cells by 25 %.

The rapidity of expression of the toxic effect of cereulide and valinomycin on the PBLs is probably due to the high lipophilicity of these toxins, their log K_{ow}:s being 5.99 and 7.46, respectively [14]. Such high lipophilicity allows the toxins to penetrate immediately through biological membranes. Other well-known lipophilic toxic compounds are DDT (di(para-chloro-phenyl)-trichloroethane) and PCB (polychlorinated biphenyls), with K_{ow} values ranging from 4.3 to 8.3 depending on the congener [86].

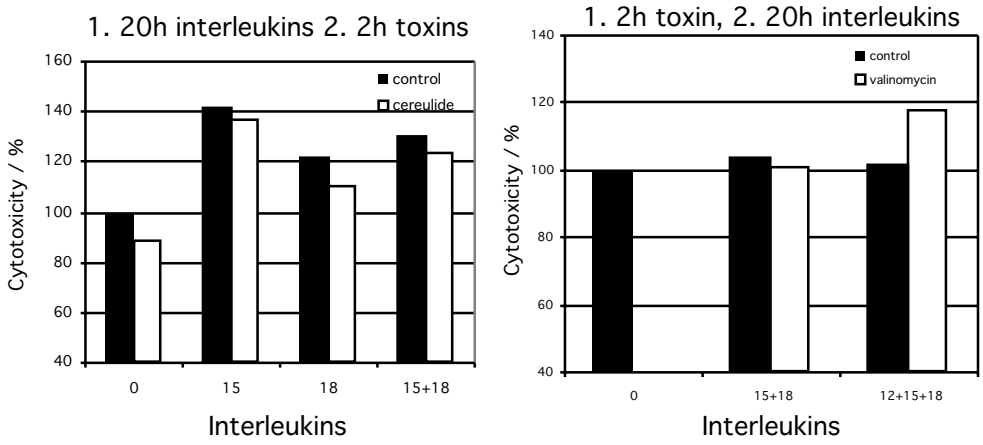


Figure 9. Effects of valinomycin and of cereulide with interleukins on the NK cell cytotoxicity. A. Treatment of interleukins 20 h followed by 2 h toxin (cereulide) exposure; B. 2 h exposure to the toxin (valinomycin) followed by 20 h interleukin treatment (n = 4).

The heterogeneity of NK cells (Figure 12) [41, 87] may explain the fact that some cytotoxicity was always detectable after the toxin treatments. An alternative explanation for the residual cytotoxicity may be that the cytotoxic machinery of NK cells became resistant to the toxins at some activation level. This activation level may vary depending on the infection status of the host. The interleukin influence on NK cell cytotoxicity is shown in Figure 8, where interleukin treatment before or after toxin treatment recovered the cytotoxicity to a level as high as the original cytotoxicity.

Yet another explanation for the residual cytotoxicity is that small in concentrations of toxins are not distributed evenly to all NK cells. This, however, is unlikely since even very high concentrations of the toxins did not eradicate the residual cytotoxicity, as seen in Figure 1, Paper II.

4.2.2 Cereulide effects

The present results in this thesis show that exposure to cereulide, the emetic toxin of *Bacillus cereus*, partially inhibited the cytotoxic activity of NK cells. As was in the case of valinomycin, the kinetics of the NK cell cytotoxicity inhibition by cereulide was fast - it occurred within the first minute of exposure. The inhibition was clear and remained stabile for the following 3 hours. Data obtained during the 20 minute exposures are shown in Figure 2b, Paper III.

The used cereulide preparations were purified from two different strains of *Bacillus cereus* (F5881/94 and F4810/72). The effects of cereulide purified from these strains were compared to those of valinomycin, which was included in the study as a reference toxin. The cytotoxicity of PBLs was inhibited by both preparations of cereulide, the level of inhibition being slightly lower than that observed for valinomycin.

The difference in the inhibiting effects of cereulide and valinomycin may rely on the structure of cereulide, which differs from that of valinomycin. Variation between individual NK cell donors was also observed (Paper III, Figure 2a). In general, however, the results were very similar to those obtained by valinomycin.

4.3 Morphological changes in toxin treated NK cells

Valinomycin, as well as cereulide, induced distinct morphological changes in exposed NK cells. Both toxins provoked the formation of large cytoplasmic vacuoles in NK cells. This was easy to see by different microscopic techniques. We used ordinary light microscopy and fluorescence microscopy, as well as scanning (SEM) and transmission (TEM) electron microscopies.

Microscopic pictures showed that NK cells treated with valinomycin or with cereulide displayed characteristic vacuoles, which were not observed in untreated NK cells (Figure 10).

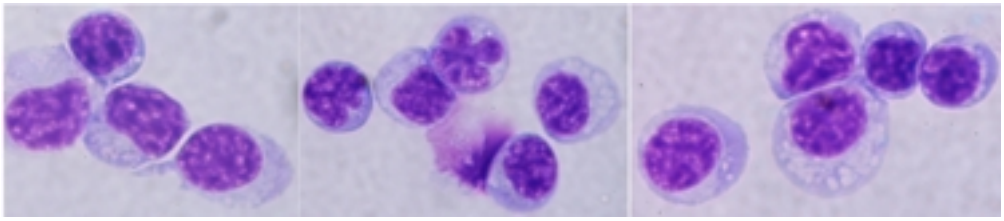


Figure 10. Light microscopic pictures of Giemsa stained NK cells. A. Unexposed cells, B. cells exposed for 2 h at 37°C to valinomycin, 1000 ng/ml, C. to cereulide, 50 ng/ml , 2 h at 37°C.

Details of the vacuoles visible in the Giemsa stained NK cells, were examined with SEM and TEM techniques. In SEM picture untreated NK cell displays typical surface projections of the NK cells. The surface of a toxin-treated cell is different: the surface projections were shorter and thicker, as demonstrated in Figure 3, Paper II. Cereulide also induced similar changes on the NK cell surface (data not shown).

TEM pictures of the resting NK cells showed a large nucleus and several intact mitochondria, Figure 2, Paper II and Figure 5, Paper III. An exposure of 3 h to valinomycin or to cereulide induced the swelling of mitochondria and distortion of mitochondrial cristae and of the inner membrane of purified NK cells and cells of the NK-92 cell line. Nuclear changes were minimal, only mild condensation of chromatin was seen (Figure 11 in this thesis shows only mitochondrial changes)

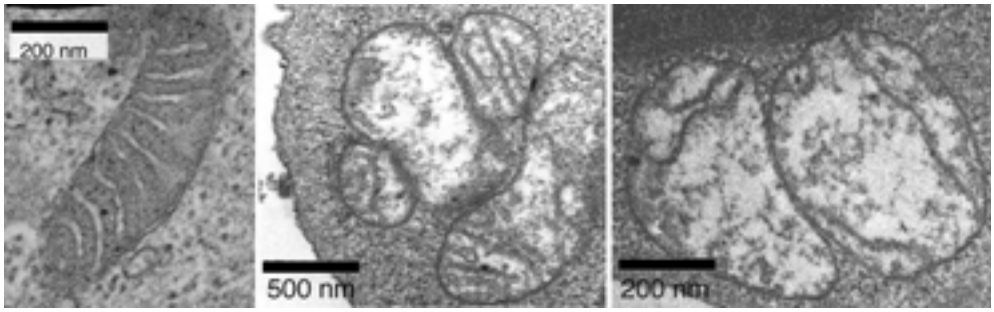


Figure 11. TEM pictures of NK cell mitochondria. A. An intact mitochondrion of NK cell. Mitochondria of B. valinomycin (100 ng/ml) and C. cereulide (100 ng/ml) exposed NK cells.

4.4 Mitochondrial transmembrane potential changes in toxin treated cells

NK cells were stained with the lipophilic dye JC-1, which displays the changes of the mitochondrial membrane potential ($\Delta\psi_m$). Normal mitochondria are energized and $\Delta\psi_m$ is high. The high $\Delta\psi_m$ in JC-1 stained cells is visible as a yellow fluorescence of mitochondria, whereas the cytoplasmic membrane potential is lower and the fluorescence green. When the $\Delta\psi_m$ decreases, the intensity of the yellow fluorescence of the mitochondria decreases or disappears.

Unexposed JC-1-stained human NK cells presented the fluorescence pattern displayed in Figure 12A in this thesis and in Figure 4, Paper III. NK cells exposed to a small dose (0.1 ng/ml) of cereulide displayed a decreased intensity of yellow fluorescence emission. A high dose of cereulide, 500 ng/ml, caused the total disappearance of yellow fluorescence, leaving the cells green, indicating the low $\Delta\psi_m$ in Figure 4, Paper III.

Interestingly, when studying large numbers of NK cells it was later observed that a minority of NK cells did not lose the mitochondrial $\Delta\psi_m$ in response to the toxin treatment. This was demonstrated in fluorescence microscopy pictures taken of NK cells exposed to 12.5 and 100 ng/ml valinomycin, in Figure 12. Those individual cells are possibly responsible for the residual killing and production of cytokines in assays, as described earlier in paragraphs 4.2.1 and 4.2.2.

Our attempt to phenotype the toxin resistant NK cells was unsuccessful. It will be interesting to study sensitivity of $CD56^{bright}$ and $CD56^{dim}$ NK cells to these toxins since these subpopulations differ dramatically in cytotoxic capacity and IFN-γ production [87].

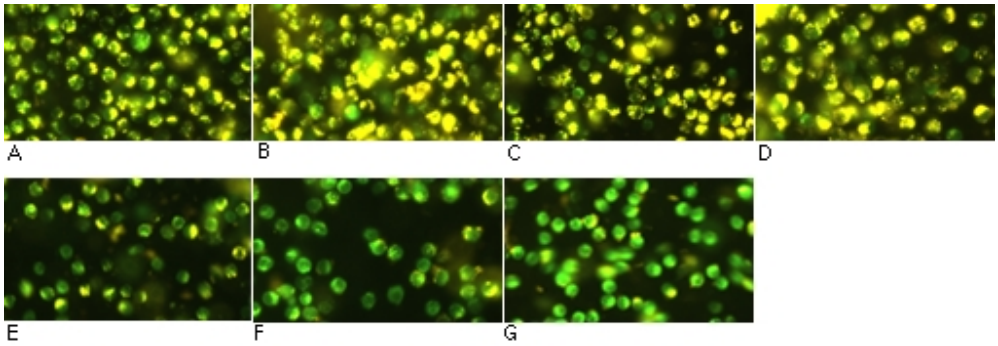


Figure 12. NK cells stained with JC-1 and viewed with a fluorescence microscope. A. Resting NK cells. Cells exposed to valinomycin at concentrations of: B. 1 ng/ml, C. 3.1 ng/ml, D. 6.3 ng/ml, E. 12.5 ng/ml, F. 25 ng/ml, and G. 100 ng/ml.

When Hep G2 (hepatoblastoma) and HeLa (adenocarcinoma from cervix) cell lines and the resting human NK cell were compared, the NK cells proved most sensitive to valinomycin exposure as measured by loss of mitochondrial transmembrane potential (Figure 13A). The NK-92 cell line, which resembles normal NK cells, was almost as sensitive to the toxic effect. HepG2 and HeLa represent parenchymic epithelial cells and were more resistant to valinomycin.

These results reveal the disadvantage of the use of cultured cell lines in toxicity testing: cell lines do not necessarily reflect the actual toxicity of various compounds *in vivo*. Activation of NK cells with different combinations of interleukins did not protect the cells against collapse of the mitochondrial membrane potential.

4.5 Apoptosis in NK cells

To assess the role of caspase activity in apoptosis, a general caspase-inhibitor, Z-VAD-FMK, and a specific caspase-3 inhibitor, Z-DEVD-FMK were used. Apoptosis was measured by annexinV-FITC/ propidium iodide stainings, flow cytometry, caspase-3 activity assays, and DNA fragmentation analyses.

4.5.1 Effects of valinomycin and cereulide

NK cells were found to be highly sensitive to the valinomycin and cereulide induced apoptosis when compared to T cells. These results are described in Papers II, Figure 4 and III, Figure 6. The data presented in Papers II and III include both early and late apoptosis. After 2 h of toxin treatment the apoptosis rate was measured by AnnexinV-FITC. The proportion of apoptotic NK cells did not deviate from the control level within 2 hours. The toxin effects appeared after 24 h, when the ratio of apoptotic cells was double that of untreated NK cells. The ratio of early and late apoptotic cells continued to increase during the subsequent two days. At the same time similarly treated T cells showed only a slight increase of apoptosis. These results are shown in Figure 4, Paper II and in Figure 6, Paper III.

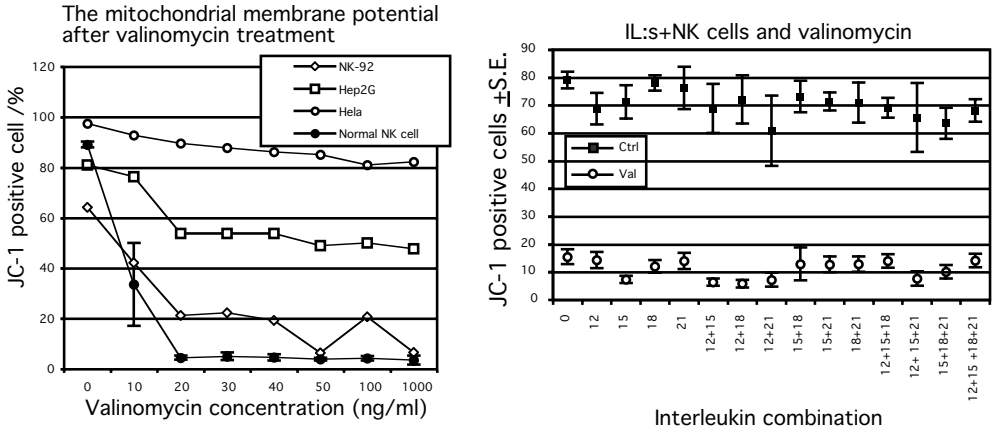


Figure 13. Flow cytometric analysis of changes in the mitochondrial membrane potential of human cell lines and resting NK cells in response to1 h valinomycin exposure. Membrane potential was detected as yellow fluorescence of cells stained with JC-1. A. The effect of valinomycin concentrations on the mitochondrial membrane potential of different cell types. These NK cell values represent the average (± S.E.) from 7 individual donors. B. Effects of 1h valinomycin (20 ng/ml) exposure on mitochondrial membrane potentials. The figure shows results obtained with resting NK cells, pre-incubated overnight with different combinations of interleukins. These values represent averages (± S.E.) from 10 individual donors.

These results further suggest that NK cells are highly sensitive to the toxic effects of cereulide and valinomycin, and therefore these toxins primarily damage the first line of immune defence.

4.5.2 Role of caspase-3

The majority of known apoptotic pathways are caspase-3 dependent. Therefore the caspase-3 activity of valinomycin-treated and untreated NK and T cells was measured. Interleukin-stimulated NK cells were also included because the earlier results indicated that interleukins may protect some NK cell functions against toxin attack. The mitochondrial changes observed in the TEM pictures suggested that caspase-3-dependent apoptosis (see the Introduction, caspase-part) could occur in NK cells. (Figure 2, Paper II and Figure 5, Paper III).

The results obtained with isolated resting NK cells revealed a high caspase-3 activity in the absence of activation, thus differing in this respect from T cells [71]. Treatment of NK cells with the toxin or interleukin also left the caspase-3 activity unaffected. After 24 hour culturing, the caspase-3 activity of NK cells decreased, as described in Figure 1, Paper IV. The general caspase inhibitor Z-VAD-FMK totally inhibited

caspase-3 activity. (Figure 1, Paper IV). However, flow cytometric analysis of Z-VAD-FMK and/or valinomycin-treated NK cells showed that general caspase inhibitor could not prevent early or late apoptosis nor necrosis. These results suggested that a caspase-3-independent pathway of apoptosis exists in NK cells (Figure 2, Paper IV).

However, somewhat contradicting results were obtained from the DNA analyses. One marker of apoptosis is the subgenomic DNA peak, subG1. The subG1 analysis of valinomycin-treated NK cells indicated that caspase inhibitor partly inhibited valinomycin- induced NK cell apoptosis. In the presence of general caspase inhibitor, the subG1 peak decreased from 22 % to only 9 %, shown in Figure 6, Paper IV. Interestingly, DNA ladder analysis [88] further showed that inhibition of caspases prevented the ladder formation neither in valinomycin treated nor in anti-Fas induced NK cells as shown in Figure 6, Paper IV. Taken together, these results suggest that valinomycin induces in NK cells two types of apoptosis simultaneously; one caspase-3-dependent and one caspase-3-independent. The caspase-3 independent apoptosis could, for instance, be endo-G-mediated (Figure 7) [79]. When valinomycin induced clear mitochondrial damages, it is possible that the endo-G was released from the mitochondrial matrix and caused the ladder formation of DNA. The same phenomenon likely occurs with cereulide.

4.6 Other effects of toxins on NK cells

Although the exact mechanism of NK cell cytotoxicity inhibition by the toxins is not clear, several explanations can be suggested on the basis of the current results. One explanation is the toxin-induced inhibition of ATP production, at least partly caused by disruption of mitochondria (Table 1, Paper IV). The shrinking reservoir of ATP may hamper the operation of the cytotoxic apparatus and the mobility of NK cells. Another mechanism of inhibition is simply the reduction of effector cell numbers through apoptosis. When the effect of general caspase inhibitor Z-VAD-FMK was tested on NK cell proliferation, IFN-γ production, and cytotoxicity, a modest increase in these activities was detected, as described in Paper IV. These observations suggest that caspase-3 has only a restricted role in NK cell biology; it is most probably involved in the control of NK cell life span and homeostasis [89]. This may function as part of the immune system to avoid autoimmune diseases.

4.7 Possible dangers of human exposure to potassium ionophores

Cereulide exposure usually comes from food. Improper conditions of food storage may permit cereulide production in food, thus increasing the risk of cereulide exposure. These foods are usually nonrefrigerated and stored under conditions that allow moisture retention (i.e. a plastic bag). Under such conditions even a low contamination by cereulide producing *B. cereus* may also result in levels of cereulide harmful to consumer's health. The cereulide dose, which causes acute emetic disease in human is ≤ 8 µg/kg body weight [90]. Toxin risks are associated with reheated foods, meals, or meal residues stored nonrefrigerated for hours or days, for instance pasta, vegetables, and steamed or cooked rice. Furthermore, the presently applied shelf life of

bakery products ranges from 4 to 75 days (depending on the type of product). Recently, cereulide-producing *B. cereus* strains were also found in indoor dust and indoor materials of moisture-affected buildings [91], indicating an exposure route by inhalation.

The present study showed that the NK cells capacity to kill abnormal cells is impaired after toxin exposure. If such exposure is long lasting, as may occur in moisture damaged buildings, the impaired NK function may lead to a compromised immune defence towards microbial infections and cancer.

In industrialized countries human exposure to the other *Streptomyces* produced potassium ionophores lasalocid, salinomycin, and narasin (Table 1) is likely to occur, since these substances are used on a large scale as animal feed additives (Table 2). In Finland, the annual usage of these substances in animal feeds averaged during 1000 kg to 6000 kg in 1996 to 2002 [11]. Human exposure may result from residues remaining in foodstuffs of animal origin [8, 13] and it may occur in occupational situations by air or skin contact (animal feed handlers and livestock farmers). In a boar spermatozoan assay for mitochondrial toxicity, lasalocid was found to be similarly toxic as cereulide and valinomycin. Salinomycin and narasin were also toxic to mitochondria but less potent (D. Hoornstra, unpublished information). Since these substances are also lipophilic, and thus likely to passively permeate into tissues and cells, effects on immune cells may expected to be similar to those observed for valinomycin and cereulide in the present thesis.

5. Conclusions

5.1 IFN-α and IL-18 enhance IFN-γ production by purified resting human NK cells in a synergistic manner. Additionally, the combination of IL-12 and IL-18 induces high IFN-γ production. IL-12, IL-15 and IL-18 are synergistic in the induction by IFN-γ and GM-CSF production by NK cells. The production IFN-γ and GM-CSF by NK cells is inhibited by two environmental toxins, valinomycin and cereulide. Cereulide is slightly less toxic than valinomycin.

5.2 Valinomycin and cereulide are more toxic to NK cells than to peripheral blood T cells and monocytes.

5.3 Valinomycin partially inhibits the cytotoxicity of NK cells, and the kinetics of the effect is very rapid. Also cereulide inhibits partially and very quickly the cytotoxicity of NK cells — very similar to valinomycin.

5.4 Valinomycin and cereulide induce mitochondrial swelling of NK cells.

5.5 A minority of NK cells do not lose the mitochondrial transmembrane potential upon toxin treatment. These residual cells are cytotoxic and produce cytokines even after toxin exposure. NK cells lost mitochondrial membrane potential at exposure to lower valinomycin concentrations as compared with other human cell types.

5.6 Valinomycin and cereulide induce apoptosis of NK cells. The apoptotic effect on T cells is much weaker.
Valinomycin exposure does not affect the caspase-3 activity of NK cells and T cells.
Valinomycin induces simultaneously both caspase-3-dependent and caspase-3-independent apoptosis in NK cell.

5.7 Humans can be exposed to various microbially produced potassium ionophores derived from food or environment.

The present study concentrated on two major topics. Firstly, what are the responses induced by two K^+ ionophoric toxins, valinomycin and cereulide, on NK cell viability and functions. Secondly, how IFN-α and different interleukin combinations affect the IFN-γ production by NK cell.

NK cells represent the first line of immune defence and therefore the impairment of optimal NK cell functions, shown in the present study, may subsequently lead to a compromised defence against microbial invasion and/or neoplastic disease. Diminished IFN-γ production by NK cells, due to toxin action, also may enhance hypersensitivity reactions. As compared with other cells of the immune system, NK cells were particularly sensitive to the tested potassium ionophoric toxins. NK cytotoxicity assay should be considered as a tool for investigating toxins potentially encountered in the environment and/or in food.

6. Acknowledgements

This work was carried out at the Department of Pathology, Haartman Institute and the Department of Applied Chemistry and Microbiology, Division of Microbiology, University of Helsinki.

This work was financially supported by the Sigrid Juselius Foundation, the Helsinki University Central Hospital, the Finnish Konkordia Union, the Food Research Foundation, the Finnish Work Environment fund, Ida Montin Foundation, Väinö and Laina Kivi Foundation, Academy of Finland (grant 50733), the Microbial Resources Research Unit and the Finnish Graduate School of Applied Biosciences (ABS).

I want to express my warmest thanks to: My supervisors professor Mirja Salkinoja-Salonen and docent Tuomo Timonen. Especially for Mirja's positive attitude and support during this work, and Tuomo's professional knowledge of NK cells.

I wish to thank professors Eero Saksela and Veli-Pekka Lehto, the previous and the current heads of Department of Pathology, for the possibility of using the working facilities of the department.

Professors Mikael Skurnik and Olli Silvennoinen are gratefully thanked for reviewing the manuscript of this thesis and the constructive criticism, which improved the thesis.

My co-workers Maria Andersson and Raimo Mikkola helped always with the bacterial toxins and JC-1 stainings. Timo Sareneva, Sampsa Matikainen, Ilkka Julkunen, and Minja Miettinen kindly helped with the cytokine problems and taught the ELISA method for me. I also thank Michael Hess for the electron microscopy skills. Tuula Helander, Päivi Majander-Nordenswan, Hilkka Lankinen, Antti Salminen and Ari Ora, whose always had time to think about life and science and Ari, of course, computer problems. Erkki Hölttä and Kristiina Järvinen, whose had time to think about my problems with the last article. Douwe Hoornstra is thanked for allowing me to cite unpublished results. And of course Lauri Ylimaa, who kindly helped with pictures and made the layout of this thesis.

For their excellent assistance, help, support and friendship of Leena Harju, Leena Saikko, Monica Shoultz, Taina Nieminen and Johanna Soikkeli.

Good friends were also needed, Taina, Johnny, Sari, Ritva and Gunilla and her daughter Hilda were always giving a helping hand when needed.

And I also thank my family. During these years I always received help and support from my parents Tuula and Leo Paananen, my mate Jaakko and his son Riku Virtanen, and last but not least my kids Johan and Sofia Engman, whose lives were not the easiest at home during this sometimes hard work.

7. Literature cited

1. MA Andersson, R Mikkola, RM Kroppenstedt, FA Rainey, J Peltola, J Helin, K Sivonen, MS Salkinoja-Salonen (1998): The mitochondrial toxin produced by *Streptomyces griseus* strains isolated from an indoor environment is valinomycin. Appl Environ Microbiol 64:4767-73.

2. MS Salkinoja-Salonen, MA Andersson, R Mikkola, A Paananen, J Peltola, H Mussalo-Rauhamaa, R Vuorio, N-E Saris, P Grigorjev, J Helin, U Koljalg, T Timonen (1998): Toxigenic microbes in indoor environment: identification, structure and biological effects of the aerosolizing toxins. In: 3rd Int. Conf. On Bioaerosols, Fungi and Mycotoxins. E. Johanning, ed., Mount Sinai Press, NY., Saratoga Springs NY, USA, 629.

3. D Nelson, M Cox (2000): Lehningers priciples of biochemistry, Worth Publishers, New York, 417-433.

4. B Alberts, A Johnson, J Lewis, M Raff, K Roberts, P Walter (2002): Molecular biology of the cell, Garland Science, New York (NY), 625-655.

5. L Stryer (2002): Biochemistry, W.H. Freeman and Company, New York, 355-363.

6. U Gräfe (1992): Biochemistry of Antibiotics. Biochemie der Antibiotika, Spektrum Akademischer Verlag GmbH, Heidelberg, Berlin, New York, 124-282.

7. D Hoornstra, MA Andersson, R Mikkola, MS Salkinoja-Salonen (2003): A new method for in vitro detection of microbially produced mitochondrial toxins. Toxicol In Vitro 17:745-751.

8. P Butaye, LA Devriese, F Haesebrouck (2003): Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. Clin Microbiol Rev 16:175-188.

9. TM Devlin (2002): Textbook of biochemistry with clinical correlations, Wiley-Liss, New York, 522-531.

10. V Henriksen (2002): Residues of Veterinary Drugs in Foodstuffs 2001 - Results from the Danish Survey on Drug Residues. Institute of Food Safety and Nutrition, Department of Chemical Contaminants. Veterinære lægemiddelrester i fødevarer 2001 - resultater fra den danske kontrol med veterinære lægemiddelrester, Institut for Fødevaresikkerhed og Ernæring, Afdelingen for Kemiske Forureninger, Søborg, Denmark, 1-46. Access date 11.11.03 http://www.vfd.dk/FDir/Publications/2002023/Rapport.pdf.

11. Anonym (1999 and 2003): Use of Antimicrobial Additives in Animal feeds. Antimikrobisten rehun lisäaineiden käyttö eläimillä, Center for Crop Inspection. Kasvintuotannon tarkastuskeskus, Helsinki, Finland.

12. Anonym (1998): Antibiotic Use in U.S. Livestock Production, The Animal and Plant Health inspection Service (APHIS) of U.S. Department of Agriculture's (USDA). Access date 18.11.03 http://www.aphis.usda.gov/vs/ceah/cei/antiresist.antibiouse.pdf.

13. Anonym (2003): Results of residue examinations of products of animal origin in Finland 2002. Eläimistä saatavien elintarvikkeiden vierasainetutkimukset 2002, National Food Agency (EVI), National Veterinary and Food Research Institute (EELA), Ministry of Agriculture and Forestry (MMM), Helsinki, 1-68. Acces date 11.11.03, http://www.elintarvikevirasto.fi/

14. R Mikkola, NE Saris, PA Grigoriev, MA Andersson, MS Salkinoja-Salonen (1999): Ionophoretic properties and mitochondrial effects of cereulide: the emetic toxin of *B. cereus*. Eur J Biochem 263:112-117.

15. JB Perkins, SK Guterman, CL Howitt, VE Williams, 2nd, J Pero (1990): Streptomyces genes involved in biosynthesis of the peptide antibiotic valinomycin. J Bacteriol 172:3108-116.

16. L Stryer (1995): Biochemistry, Freeman, New York, 260-274.

17. WL Duax, JF Griffin, DA Langs, GD Smith, P Grochulski, V Pletnev, V Ivanov (1996): Molecular structure and mechanisms of action of cyclic and linear ion transport antibiotics. Biopolymers 40:141-155.

18. S Pitchayawasin, M Kuse, K Koga, M Isobe, N Agata, M Ohta (2003): Complexation of cyclic dodecadepsipeptide, cereulide with ammonium salts. Bioorg Med Chem Lett 13:3507-3512.

19. IJ Furlong, C Lopez Mediavilla, R Ascaso, A Lopez Rivas, MK Collins (1998): Induction of apoptosis by valinomycin: mitochondrial permeability transition causes intracellular acidification. Cell Death Differ 5:214-221.

20. RC Duke, RZ Witter, PB Nash, JD Young, DM Ojcius (1994): Cytolysis mediated by ionophores and pore-forming agents: role of intracellular calcium in apoptosis. Faseb J 8:237-246.

21. Y Inai, M Yabuki, T Kanno, J Akiyama, T Yasuda, K Utsumi (1997): Valinomycin induces apoptosis of ascites hepatoma cells (AH-130) in relation to mitochondrial membrane potential. Cell Struct Funct 22:555-563.

22. YA Ovchinnikov (1974): Second FEBS-Ferdinand Springer lecture: Membrane active complexones. Chemistry and biological function. FEBS Lett 44:1-21.

23. D Radcliffe (1997): Valinomycin molecule. Access date 19.11.2003 http://molsim.ch.umist.ac.uk/vm.shtml

24. N Agata, M Mori, M Ohta, S Suwan, I Ohtani, M Isobe (1994): A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells. FEMS Microbiol Lett 121:31-34.

25. N Agata, M Ohta, M Mori, M Isobe (1995): A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. FEMS Microbiol Lett 129:17-20.

26. N Agata, M Ohta, M Mori (1996): Production of an emetic toxin, cereulide, is associated with a specific class of *Bacillus cereus*. Curr Microbiol 33:67-69.

27. PE Granum, T Lund (1997): *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol Lett 157:223-228.

28. A Kotiranta, K Lounatmaa, M Haapasalo (2000): Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes Infect 2:189-198.

29. SH Beattie, AG Williams (1999): Detection of toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. with an improved cytotoxicity assay. Lett Appl Microbiol 28:221-225.

30. RT Briley, JH Teel, JP Fowler (2001): Nontypical *Bacillus cereus* outbreak in a child care center. J Environ Health 63:9-11.

31. N Agata, M Ohta, K Yokoyama (2002): Production of *Bacillus cereus* emetic toxin (cereulide) in various foods. Int J Food Microbiol 73:23-27.

32. WJ Finlay, NA Logan, AD Sutherland (2000): *Bacillus cereus* produces most emetic toxin at lower temperatures. Lett Appl Microbiol 31:385-389.

33. EL Jääskelainen, MM Haggblom, MA Andersson, L Vanne, MS Salkinoja-Salonen (2003): Potential of *Bacillus cereus* for producing an emetic toxin, cereulide, in bakery products: quantitative analysis by chemical and biological methods. J Food Prot 66:1047-1054.

34. H Mahler, A Pasi, JM Kramer, P Schulte, AC Scoging, W Bar, S Krahenbuhl (1997): Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. N Engl J Med 336:1142-1148.

35. K Shinagawa, H Konuma, H Sekita, S Sugii (1995): Emesis of rhesus monkeys induced by intragastric administration with the HEp-2 vacuolation factor (cereulide) produced by *Bacillus cereus*. FEMS Microbiol Lett 130:87-90.

36. K Shinagawa, Y Ueno, D Hu, S Ueda, S Sugii (1996): Mouse lethal activity of a HEp-2 vacuolation factor, cereulide, produced by *Bacillus cereus* isolated from vomiting-type food poisoning. J Vet Med Sci 58:1027-1029.

37. K Yokoyama, M Ito, N Agata, M Isobe, K Shibayama, T Horii, M Ohta (1999): Pathological effect of synthetic cereulide, an emetic toxin of *Bacillus cereus*, is reversible in mice. FEMS Immunol Med Microbiol 24:115-120.

38. MA Andersson, U Koljalg, R Mikkola, J Peltola, R Vuorio, MS Salkinoja-Salonen (1999). Myrkylliset mikrobit sisätiloissa. Toxic indoor microbes. In: Mikrobiologian julkaisuja. M. S. Salkinoja-Salonen, ed., Soveltavan kemian ja mikrobiologia laitos, Helsingin Yliopisto, Helsinki, 1-46.

39. N Sakurai, KA Koike, Y Irie, H Hayashi (1994): The rice culture filtrate of *Bacillus cereus* isolated from emetic-type food poisoning causes mitochondrial swelling in a HEp-2 cell. Microbiol Immunol 38:337-343.

40. G Trinchieri (1989): Biology of natural killer cells. Adv Immunol 47:187-376.

41. LE Jonges, P Albertsson, RL van Vlierberghe, NG Ensink, BR Johansson, CJ van de Velde, GJ Fleuren, U Nannmark, PJ Kuppen (2001): The phenotypic heterogeneity of human natural killer cells: presence of at least 48 different subsets in the peripheral blood. Scand J Immunol 53:103-110.

42. WE Seaman (2000): Natural killer cells and natural killer T cells. Arthritis Rheum 43:1204-1217.

43. EO Long, DF Barber, DN Burshtyn, M Faure, M Peterson, S Rajagopalan, V Renard, M Sandusky, CC Stebbins, N Wagtmann, C Watzl (2001): Inhibition of natural killer cell activation signals by killer cell immunoglobulin-like receptors (CD158). Immunol Rev 181:223-233.

44. WM Yokoyama, BF Plougastel (2003): Immune functions encoded by the natural killer gene complex. Nat Rev Immunol 3:304-16.

45. M Cavalcanti, A Jewett, B Bonavida (1999): Irreversible cancer cell-induced functional anergy and apoptosis in resting and activated NK cells. Int J Oncol 14:361-366.

46. LL Lanier (2000): The origin and functions of natural killer cells. Clin Immunol 95:S14-18.

47. CA Biron (1997): Activation and function of natural killer cell responses during viral infections. Curr Opin Immunol 9:24-34.

48. JH Curfs, JF Meis, JA Hoogkamp-Korstanje (1997): A primer on cytokines: sources, receptors, effects, and inducers. Clin Microbiol Rev 10:742-780.

49. M Gadina, PR Ferguson, JA Johnston (2003): New interleukins: are there any more? Curr Opin Infect Dis 16:211-217.

50. JA Gracie, SE Robertson, IB McInnes (2003): Interleukin-18. J Leukoc Biol 73:213-224.

51. J Parrish-Novak, SR Dillon, A Nelson, A Hammond, C Sprecher, JA Gross, J Johnston, K Madden, W Xu, J West, S Schrader, S Burkhead, M Heipel, C Brandt, JL Kuijper, J Kramer, D Conklin, SR Presnell, J Berry, F Shiota, S Bort, K Hambly, S Mudri, C Clegg, M Moore, FJ Grant, C Lofton-Day, T Gilbert, F Rayond, A Ching, L Yao, D Smith, P Webster, T Whitmore, M Maurer, K Kaushansky, RD Holly, D Foster (2000): Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature 408:57-63.

52. P Sheppard, W Kindsvogel, W Xu, K Henderson, S Schlutsmeyer, TE Whitmore, R Kuestner, U Garrigues, C Birks, J Roraback, C Ostrander, D Dong, J Shin, S Presnell, B Fox, B Haldeman, E Cooper, D Taft, T Gilbert, FJ Grant, M Tackett, W Krivan, G McKnight, C Clegg, D Foster, KM Klucher (2003): IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 4:63-68.

53. S Romagnani (1997): The Th1/Th2 paradigm. Immunol Today 18:263-266.

54. DO Gor, NR Rose, NS Greenspan (2003): TH1-TH2: a procrustean paradigm. Nat Immunol 4:503-505.

55. CA Biron, L Brossay (2001): NK cells and NKT cells in innate defense against viral infections. Curr Opin Immunol 13:458-464.

56. L Rogge, D D'Ambrosio, M Biffi, G Penna, LJ Minetti, DH Presky, L Adorini, F Sinigaglia (1998): The role of Stat4 in species-specific regulation of Th cell development by type I IFNs. J Immunol 161:6567-6574.

57. T Sareneva, S Matikainen, M Kurimoto, I Julkunen (1998): Influenza A virus-induced IFN-alpha/beta and IL-18 synergistically enhance IFN-gamma gene expression in human T cells. J Immunol 160:6032-6038.

58. S Ushio, M Namba, T Okura, K Hattori, Y Nukada, K Akita, F Tanabe, K Konishi, M Micallef, M Fujii, K Torigoe, T Tanimoto, S Fukuda, M Ikeda, H Okamura, M Kurimoto (1996): Cloning of the cDNA for human IFN-gamma-inducing factor, expression in Escherichia coli, and studies on the biologic activities of the protein. J Immunol 156:4274-4279.

59. JA Hamilton (2002): GM-CSF in inflammation and autoimmunity. Trends Immunol 23:403-408.

60. R Hunt (2003): γ-IFN secretion, The Board of Trustees of the University of South Carolina. Access date 181103, last modified 240903, http://www.med.sc.edu/micro/book/immunol-sta.htm

61. G Trinchieri (2003): Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 3:133-146.

62. CL Karp, M Wills-Karp (2001): Complement and IL-12: yin and yang. Microbes Infect 3:109-119.

63. TA Fehniger, MH Shah, MJ Turner, JB VanDeusen, SP Whitman, MA Cooper, K Suzuki, M Wechser, F Goodsaid, MA Caligiuri (1999): Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. J Immunol 162:4511-4520.

64. BR Lauwerys, JC Renauld, FA Houssiau (1999): Synergistic proliferation and activation of natural killer cells by interleukin 12 and interleukin 18. Cytokine 11:822-830.

65. TA Fehniger, MA Caligiuri (2001): Interleukin 15: biology and relevance to human disease. Blood 97:14-32.

66. J Parrish-Novak, DC Foster, RD Holly, CH Clegg (2002): Interleukin-21 and the IL-21 receptor: novel effectors of NK and T cell responses. J Leukoc Biol 72:856-863.

67. MA Cooper, JE Bush, TA Fehniger, JB VanDeusen, RE Waite, Y Liu, HL Aguila, MA Caligiuri (2002): In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. Blood 100:3633-3638.

68. M Prlic, BR Blazar, MA Farrar, SC Jameson (2003): In vivo survival and homeostatic proliferation of natural killer cells. J Exp Med 197:967-976.

69. M Strengell, S Matikainen, J Siren, A Lehtonen, D Foster, I Julkunen, T Sareneva (2003): IL-21 in Synergy with IL-15 or IL-18 Enhances IFN-gamma Production in Human NK and T Cells. J Immunol 170:5464-5469.

70. NA Maianski, D Roos, TW Kuijpers (2003): Tumor necrosis factor alpha induces a caspase-independent death pathway in human neutrophils. Blood 101:1987-1995.

71. C Miossec, V Dutilleul, F Fassy, A Diu-Hercend (1997): Evidence for CPP32 activation in the absence of apoptosis during T lymphocyte stimulation. J Biol Chem 272:13459-13462.

72. RC Budd (2001): Activation-induced cell death. Curr Opin Immunol 13:356-362.

73. M Jäättela (2002): Programmed cell death: many ways for cells to die decently. Ann Med 34:480-488.

74. S Nagata (2000): Apoptotic DNA fragmentation. Exp Cell Res 256:12-18.

75. C Cande, I Cohen, E Daugas, L Ravagnan, N Larochette, N Zamzami, G Kroemer (2002): Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie 84:215-222.

76. M Jäättela, J Tschopp (2003): Caspase-independent cell death in T lymphocytes. Nat Immunol 4:416-423.

77. S Pandey, B Smith, PR Walker, M Sikorska (2000): Caspase-dependent and independent cell death in rat hepatoma 5123tc cells. Apoptosis 5:265-275.

78. C Cande, F Cecconi, P Dessen, G Kroemer (2002): Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? J Cell Sci 115:4727-4734.

79. LY Li, X Luo, X Wang (2001): Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412:95-99.

80. Anonym (2003): American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland.

81. K Somersalo, E Saksela (1989): N-alpha-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) serine esterase in human cytolytic effector cells and cell line targets. Scand J Immunol 29:459-467.

82. MW Hess, MG Schwendinger, EL Eskelinen, K Pfaller, M Pavelka, MP Dierich, WM Prodinger (2000): Tracing uptake of C3dg-conjugated antigen into B cells via complement receptor type 2 (CR2, CD21). Blood 95:2617-2623.

83. JC Reed (2000): Apoptosis. In: Methods in enzymology ;. J. N. Abelson and M. I. Simon, eds., Academic Press, San Diego (CA), p.3-39.

84. I Suominen, MA Andersson, MC Andersson, AM Hallaksela, P Kampfer, FA Rainey, M Salkinoja-Salonen (2001): Toxic *Bacillus pumilus* from indoor air, recycled paper pulp, Norway spruce, food poisoning outbreaks and clinical samples. Syst Appl Microbiol 24:267-276.

85. T Sareneva, I Julkunen, S Matikainen (2000): IFN-alpha and IL-12 induce IL-18 receptor gene expression in human NK and T cells. J Immunol 165:1933-1938.

86. Anonym (2002): PCBs (Polychlorinated biphenyls) DDT (Di(para-chloro-phenyl)-tricloroetane) Dioxins. Acces date 2.1.2004, http://www.eurochlor.org/chlorine/science/risk18.pdf.

87. R Jacobs, G Hintzen, A Kemper, K Beul, S Kempf, G Behrens, KW Sykora, RE Schmidt (2001): CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. Eur J Immunol 31:3121-3127.

88. H Sakahira, M Enari, S Nagata (1998): Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96-99.

89. GM Spaggiari, P Contini, A Dondero, R Carosio, F Puppo, F Indiveri, MR Zocchi, A Poggi (2002): Soluble HLA class I induces NK cell apoptosis upon the engagement of killer-activating HLA class I receptors through FasL-Fas interaction. Blood 100:4098-4107.

90. EL Jääskelainen, V Teplova, MA Andersson, LC Andersson, P Tammela, MC Andersson, TI Pirhonen, NE Saris, P Vuorela, MS Salkinoja-Salonen (2003): In vitro assay for human toxicity of cereulide, the emetic mitochondrial toxin produced by food poisoning *Bacillus cereus*. Toxicol In Vitro 17:737-744.

91. M Andersson, R Mikkola, C Apetroaie, D Hoornstra, T Nieminen, M Salkinoja-Salonen (2002): Fungicidic and mitochondriotoxic *Bacilli* frequent in water damaged buildings. In: Indoor Air 2002 Proceedings of the 9th Conference on Indoor Air Quality and Climate. H. Levin, ed., Santa Cruz, California, 34-39.